Molecular genetic analysis of the region containing the essential *Pseudomonas aeruginosa* *asd* gene encoding aspartate- β -semialdehyde dehydrogenase

Tung T. Hoang,^{1,2} Scott Williams,² Herbert P. Schweizer^{1,3} and Joseph S. Lam^{3,4}

Author for correspondence: Herbert P. Schweizer. Tel: +1 970 491 3536. Fax: +1 970 491 1815.
e-mail: hschweizer@vines.colostate.edu

¹ Department of Microbiology, Colorado State University, Fort Collins, CO 80523, USA

² Department of Microbiology & Infectious Diseases, University of Calgary Health Sciences Center, Calgary, Alberta, Canada T2N 4N1

^{3,4} Canadian Bacterial Diseases Network³ and Department of Microbiology⁴, University of Guelph, Guelph, Ontario, Canada N1G 2W1

asd mutants of Gram-negative and some Gram-positive bacteria have an obligate requirement for diaminopimelic acid (DAP), an essential constituent of the cell wall of these organisms. In environments deprived of DAP, for example mammalian tissues, they will undergo lysis. This was previously exploited to develop vaccine strains of *Salmonella typhimurium* and cloning vectors containing *asd* as an *in vivo* selectable marker. As a first step for development of such systems for *Pseudomonas aeruginosa*, the *asd* gene from wild-type strain PAO1 was cloned by a combined approach of PCR amplification from chromosomal DNA, construction of mini-libraries and by complementation of an *Escherichia coli* Δ *asd* mutant. The nucleotide sequence of a 2433 bp *Sma*I–*Nsi*I fragment was determined. This fragment contained the C-terminal 47 nucleotides of *leuB*, encoding 3-isopropylmalate dehydrogenase; *asd*, encoding aspartate- β -semialdehyde dehydrogenase (Asd); and *orfA*, whose product showed similarity to the Asd proteins from *Vibrio* spp. By subcloning, *asd* was localized to a 1.24 kb DNA fragment which in an *E. coli* T7 expression system strongly expressed a 40 000 Da protein. The amino acid sequence was deduced from the DNA sequence. A comparison of the Asd proteins from *P. aeruginosa*, *E. coli* and *Haemophilus influenzae* revealed greater than 63% identity, demonstrating the conserved nature of Asd in Gram-negative bacteria, and defined the active-site-containing consensus sequence GGNCTVXMLMXXXLGLF as a possible signature motif. Chromosomal Δ *asd* mutants were isolated. They were auxotrophic for DAP, lysine, methionine and threonine, and lysed in the absence of DAP. Genetic analyses indicated that *orfA* probably is naturally frame-shifted and does not contribute to the Asd phenotype. By PFGE, the *asd* gene was mapped to between coordinates 1.89 and 2.15 Mbp, or 37–40 min, on the 5.9 Mbp *P. aeruginosa* chromosome.

Keywords: *Pseudomonas aeruginosa*, *asd*, chromosomal mapping, selectable marker, active site

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic pathogen causing fatal infections in debilitated and immuno-

Abbreviations: Asd, aspartate- β -semialdehyde dehydrogenase; DAP, diaminopimelic acid.

The GenBank accession number for the sequence reported in this paper is U11055.

compromised patients, especially those with granulocytopenia, severe burns and cystic fibrosis (CF) (Bodey *et al.*, 1983). The success of this organism as a pathogen can largely be attributed to a number of virulence factors, including alginate (Govan, 1988), exotoxin A (Vasil, 1986), phospholipase C (Vasil *et al.*, 1991), various proteases (Holder & Neely, 1991) and lipopolysaccharide (Cryz *et al.*, 1984). The great variety of distinct mechanisms by which these factors act and

Fig. 1. The role of *asd* in the proposed amino acid biosynthetic pathways of DAP, lysine, methionine and threonine, and in peptidoglycan synthesis.

interact to provide a survival strategy for the bacterium has been examined in several studies (for a review, see Hoiby *et al.*, 1989).

The *asd* gene encodes aspartate- β -semialdehyde dehydrogenase (Asd; EC 1.2.1.11), a key enzyme in the biosynthetic pathways for diaminopimelic acid (DAP), lysine, methionine and threonine (Fig. 1). DAP is an essential component of the peptidoglycan of all Gram-negative and some Gram-positive bacteria (Schleifer & Kandler, 1972). In the absence of DAP, *asd* mutants undergo lysis. Since DAP is absent from mammalian tissues, *asd* mutants of *Salmonella typhimurium* have been exploited to construct a balanced-lethal system for vaccine delivery vehicles using a cloned *asd* gene as the selective marker in place of antibiotic resistance markers which are impractical *in vivo* (Nakayama *et al.*, 1988). Few studies have dealt with the molecular characterization of *Pseudomonas aeruginosa* genes involved in the threonine (Clepet *et al.*, 1992), methionine (Fogliano *et al.*, 1995) and lysine (Martin *et al.*, 1986) amino acid biosynthetic pathways. The structural genes for homoserine dehydrogenase (*hom*), homoserine kinase (*thrB*) and threonine synthase (*thrC*) have been cloned, sequenced and mapped to the 10 min (*thrB*) and 31 min (*hom* and *thrC*) regions of the *P. aeruginosa* chromosome (Clepet *et al.*, 1992). Subsequently, Fogliano *et al.* (1995) showed that a direct sulphydrylation pathway is used for methionine biosynthesis from homoserine. The first step is catalysed by an O-succinylhomoserine synthase encoded by *metA*, which is located at 20 min on the chromosome, and the second step is catalysed by a O-succinylhomoserine sulphydralase encoded by *metZ*, which was mapped to the 40 min region of the chromosome. The structural gene for DAP decarboxylase, *lysA*, has also been cloned; its regulation was determined to be similar to that of *Escherichia coli lysA* (Martin *et al.*, 1986) and it was subsequently localized to the 10 min region of the chromosome (Holloway *et al.*, 1994). Despite previous trials (Clepet *et al.*, 1992), the genes encoding the enzymes catalysing the initial steps in aspartate- β -semialdehyde synthesis, *ask* (aspartokinase) and *asd* (Asd) have hitherto remained unidentified.

The aims of the present study were to clone and characterize the *asd* gene from *P. aeruginosa* in order (i) to understand the role of the gene and its product in amino acid biosynthesis in this bacterium and (ii) to allow construction of a Δ *asd* laboratory strain to facilitate development of genetic tools for *P. aeruginosa* utilizing the *asd* gene as an *in vivo* selectable marker.

METHODS

Bacterial strains and plasmids. The *E. coli* and *P. aeruginosa* strains used in this study are listed in Table 1. The plasmids utilized in this study were constructed as detailed in Table 1. The details underlying construction of pPS412 (Fig. 2) were as follows. A ~300 bp *Eco*RI biotinylated fragment from pPS410 was used to identify a ~4.5 kb chromosomal *Bam*HI-*Eco*RI fragment by genomic Southern analysis. For cloning of this DNA fragment, *Bam*HI + *Eco*RI-digested PAO1 chromosomal DNA was electrophoresed on a 1% low-gelling-point agarose gel in 0.5 × TBE (Sambrook *et al.*, 1989) and the fragments from the 3–5 kb region of the gel were eluted as described by Wieslander (1979). After ligation into *Bam*HI + *Eco*RI-digested pUC18, ampicillin-resistant (Ap^r) DH5 α F' colonies were selected and pooled. Plasmid DNA was isolated (Xiang *et al.*, 1994) and used to transform Δ *asd* *E. coli* strain SH309 to Ap resistance on LB medium without DAP supplementation. All putative Asd⁺ transformants contained the cloned 4.5 kb *Bam*HI-*Eco*RI fragment.

Growth media. LB medium (Miller, 1992) was routinely used as the rich medium for all bacterial strains. The minimal media employed were M9 (Miller, 1992) for *E. coli* and M9 or VBMM (Schweizer, 1991) for *P. aeruginosa*. For growth of *asd* mutants, the media were supplemented with 100 μ g DAP ml⁻¹ for *E. coli* and 1 mg DAP ml⁻¹ for *P. aeruginosa*. All other amino acids were present at 40 μ g ml⁻¹ and glucose was added to M9 medium as carbon source to a final concentration of 10 mM. Concentrations of antibiotics used in selection media were: *E. coli*, 100 μ g Ap ml⁻¹ and 15 μ g gentamicin (Gm) ml⁻¹; *P. aeruginosa*; 500 μ g carbenicillin (Cb) ml⁻¹ and 200–300 μ g Gm ml⁻¹.

DNA procedures. For PCR amplification of an *asd* fragment from chromosomal DNA, two oligonucleotides, P1 (5'-TGC-CAGGCGACTAC) and P2 (5'-[G/G][G/C][T/A][C/G]-GC[C/G]GCCTGGTAGGT) were used to prime synthesis from PAO1 (Holloway & Zhang, 1990) chromosomal DNA following previously described conditions (Kureishi & Bryan, 1992). The 50 μ l PCR reaction mixture contained 1 × PCR

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant properties	Reference/origin
<i>P. aeruginosa</i>		
PAO1	Prototroph	Holloway & Zhang (1990)
PAO183	PAO1 $\Delta(asd-orfA)\Omega Gm^r FRT$	This study
PAO184	PAO1 $\Delta asd::Gm^r FRT$	This study
PAO185	PAO1 $\Delta asd::\Omega Gm^r FRT$	This study
PG201	Prototroph	Ochsner <i>et al.</i> (1994)
UO606	PG201 <i>glpK::Tn5Gm</i>	Urs Ochsner, University of Colorado Health Sciences Center, CO, USA
<i>E. coli</i>		
DH5 α F'	[F ⁺ ϕ 80 <i>lacZ</i> Δ M15] $\Delta(lacZYA-argF)$ U169 <i>recA1 endA1 hsdR17</i> (<i>r_K⁻m_K⁺</i>) <i>supE44 thi-1 gyrA relA1</i>	Liss (1987)
BL21(DE3)	<i>E. coli</i> B F ⁻ <i>ompT</i> (<i>r_B⁻m_B⁻</i>)(λ DE3)	Studier <i>et al.</i> (1990)
SH309	F ⁻ <i>araD139</i> $\Delta(argF-lac)$ U169 <i>rpsL150 deoC1 ptsF25 flbB5301 rbsR relA1</i> $\Delta(mal-asd)3$ <i>ugpA704::Tn10</i>	Schweizer <i>et al.</i> (1986)
Plasmids		
pAC Ω Gm	Gm ^r , Tc ^r ; source of Gm ^r Ω fragment	Schweizer & Hoang (1995)
pBluescript SK -	Ap ^r ; multipurpose cloning vector	Stratagene
pCRII	Ap ^r ; PCR fragment TA cloning vector	Invitrogen
pEX100T	Ap ^r ; <i>sacB⁺ oriT⁺</i> ; gene replacement vector	Schweizer & Hoang (1995)
pFGS1	Ap ^r , Gm ^r ; source of Gm-resistance-conferring fragment flanked by <i>FRT</i> sites†	Laboratory collection
pMO010323	Tc ^r ; mobilizable cosmid vector; complements <i>leuB</i> and <i>asd</i>	Hoang & Schweizer (1997)
pUC18/19	Ap ^r ; multipurpose cloning vectors	Yanisch-Perron <i>et al.</i> (1985)
pUCP20/21	Ap ^r ; broad-host-range cloning derivatives of pUC18/19	West <i>et al.</i> (1994)
pUCP20T	Ap ^r ; mobilizable derivative of pUCP20	Schweizer <i>et al.</i> (1996)
pPS410	Ap ^r ; (281 bp PCR fragment cloned into pCRII)	This study
pPS412	Ap ^r ; <i>asd⁺ leuB⁺ orfA⁺</i> (4.5 kb <i>Bam</i> HI- <i>Eco</i> RI chromosomal DNA fragment cloned between same sites of pUC18; all genes are in same direction as P _{<i>lac</i>} [*])	This study
pPS452	Ap ^r ; Δasd (pPS412 with 0.95 kb <i>Sma</i> I Gm ^r fragment from pAC Ω Gm between <i>Clal</i> sites)	This study
pPS454	Ap ^r ; <i>asd⁺ leuB⁺ orfA⁺</i> (3.1 kb <i>Bam</i> HI- <i>Sph</i> I fragment from pPS412 cloned between same sites of pUC18; all genes are in opposite direction as P _{<i>lac</i>})	This study
pPS455	Ap ^r ; <i>asd⁺ orfA⁺</i> (<i>Sma</i> I deletion of pPS412)	This study
pPS456	Ap ^r ; <i>asd⁺ orfA⁺</i> (2.1 kb <i>Bam</i> HI- <i>Pst</i> I fragment from pPS412 cloned between same sites of pUC18; both genes are in same direction as P _{<i>lac</i>})	This study
pPS457	Ap ^r ; <i>asd⁺</i> [1.2 kb <i>Eco</i> RI- <i>Clal</i> (partial) fragment from pPS456 cloned between same sites of pBluescript SK -]	This study
pPS600	Ap ^r ; <i>asd⁺ leuB⁺ orfA⁺</i> (4.6 kb <i>Eco</i> RI- <i>Hind</i> III fragment from pPS412 cloned into the same sites of pUCP20T)	This study
pPS704	Ap ^r ; <i>asd⁺ leuB⁺ orfA⁺</i> (6.5 kb <i>Eco</i> RI fragment from pMO010323 cloned into <i>Eco</i> RI site of pUC18)	This study
pPS716	Ap ^r , Gm ^r ; source of Gm-resistance-conferring Ω fragment flanked by <i>FRT</i> sites†	Laboratory collection

* P_{*lac*}, *lac* operon promoter.

† *FRT*, yeast Flp recombinase target.

buffer (Promega) (50 mM Tris/HCl pH 8.0, 50 % glycerol, 100 mM NaCl, 0.1 mM EDTA, 5 mM DTT, 1 % Triton X-100), 200 μ M of each dNTP, 1.5 mM MgCl₂, 25 pmol of each primer, 100 ng chromosomal DNA and 2.5 U *Taq* DNA polymerase (Promega). The ~280 bp PCR fragment was eluted from a 2 % agarose gel in 0.5 $\times$ TBE utilizing the

SpinBind protocol (FMC Bioproducts) and ligated to the pCRII TA-cloning vector following the protocol provided by the supplier (Invitrogen). For Southern analysis, chromosomal PAO1 DNA was digested with various restriction endonucleases, electrophoresed on a 1 % agarose gel in 0.5 $\times$ TBE (Sambrook *et al.*, 1989) and transferred to Photogene nylon

Fig. 2. Restriction maps of recombinant plasmids and location of the *asd*, *leuB* and putative *orfA* genes. The + or - symbols denote the Asd phenotype conferred on Δ *asd* strain SH309 by the indicated plasmids. In all constructs except pPS454, the transcriptional orientation of *asd* is the same as the *lac* promoter. Ba, *Bam*HI; Cl, *Cla*I; Ec, *Eco*RI; Ev, *Eco*RV; Hc, *Hinc*II; Nd, *Nde*I; Ns, *Nsi*I; Ps, *Pst*I; Sa, *Sal*I; Sm, *Sma*I; Sp, *Sph*I; St, *Stu*I.

membranes (Gibco BRL) as described by Sambrook *et al.* (1989). Plasmid DNA was biotinylated by random hexamer priming following the NEBlot Phototype kit protocol (New England Biolabs). Following transfer and UV fixation (Sambrook *et al.*, 1989), the membranes were probed with the biotinylated DNA fragment according to the Phototype detection kit protocol (New England Biolabs). Nucleotide sequences were determined utilizing the *Taq* Dye Deoxy Terminator Cycle Sequencing kit and the protocol provided by the supplier (Applied Biosystems). Extensions were primed utilizing the commercially available 24 nt pUC/M13 reverse and forward sequencing primers, respectively. Labelled samples were analysed in the University of Calgary DNA Analysis Core Facility on an Applied Biosystems Model 373A DNA sequencer. Computer-assisted sequence analyses were performed utilizing the MacDNAsis (Hitachi Software Engineering), SeqEd (Applied Biosystems) and SeqVu (Garvan Medical Institute, Sydney, Australia) programs. GenBank homology searches were performed utilizing the online BLAST facilities of the National Center for Biotechnology Information at the National Library of Medicine (Bethesda, MD, USA). PROSITE searches were performed via the ExPASy World Wide Web (WWW) molecular biology server from the Geneva University Hospital and the University of Geneva, Switzerland. PFGE electrophoresis was performed according to the previously established procedure (Lightfoot & Lam, 1993). Other routine DNA methods were performed as previously described (Schweizer & Po, 1994).

Expression of plasmid-encoded polypeptides. These were overexpressed and identified with the bacteriophage T7 RNA polymerase/T7 promoter system (Studier *et al.*, 1990). Plasmids pT7-5*asd* and pT7-6*asd* were constructed by subcloning a 1.8 kb *Eco*RI-*Hind*III fragment harbouring *asd* from pPS456 (Fig. 2) into *Eco*RI+*Hind*III-cleaved pT7-5 and pT7-6 DNA (Tabor, 1994), respectively. Thus, in pT7-5*asd* transcription from the Φ 10 promoter proceeds from the *Sma*I site towards the *Cla*I sites as indicated for pPS412 in Fig. 2, and in pT7-6*asd* transcription from the Φ 10 promoter proceeds from the *Sma*I site towards the *Cla*I sites. The host strain was BL21(DE3), which contains the gene for T7 RNA polymerase under the control of the IPTG-inducible *lac*UV promoter (Studier *et al.*, 1990). The product of the cloned genes were selectively labelled with [³⁵S]methionine as previously described (Schweizer, 1991). Proteins were separated on a 0.1% SDS/10% polyacrylamide gel (pH 9.2) (Makowski & Ramsby, 1993). The destained gels were soaked in 1 M sodium salicylate (Chamberlain, 1979), dried and visualized by autoradiography.

Isolation of chromosomal *asd* mutations. A PCR-based strategy was employed to isolate chromosomal Δ *asd* mutants.

For reverse PCR amplification of fragments missing the 1111 bp *asd* coding region alone or lacking 2225 bp encompassing both the *asd* and *orfA* coding regions, but containing the respective 5' and 3' flanking sequences, three primers were designed: R3 (5'-TCGCAACTAC ACCTATATGAGAGA), which was complementary to nt 94-11 of the *asd* upstream sequence presented in Fig. 3 (the base complementary to the A of the *asd* ATG initiation codon is underlined); R1 (5'-TGATCGATCCGGCGCCGTCCGCAA), whose sequence was identical to nt 1230-1253 and includes the *asd* stop codon (underlined); and R2 (5'-GTAAAAGATACTTACCCACAGATT), which is identical to nt 2342-2365 and includes the putative full-length *orfA* stop codon (underlined). These primers were used to prime synthesis from pPS454 (Fig. 2; Table 1) DNA in a 50 μ l reaction mixture containing 1 $\times$ *Taq* Plus buffer (Sangon) (20 mM Tris/HCl pH 8.8, 10 mM KCl, 10 mM (NH₄)₂SO₄, 2 mM MgSO₄, 0.1% Triton X-100, 0.1 mg BSA ml), 200 μ M of each dNTP, 30 pmol of each primer, $\sim$ 10 pmol pPS454 DNA and 5 U *Taq* Plus polymerase (Sangon). The reaction mixtures were subjected to the following cycles: 1 cycle at 97 $^{\circ}$ C for 5 min; 30 cycles (94 $^{\circ}$ C, 1 min; 58 $^{\circ}$ C, 45 s; 72 $^{\circ}$ C, 3.5 min); final extension at 72 $^{\circ}$ C for 5 min. Combination of primers R3 and R2 [Δ (*asd*-*orfA*)] yielded a PCR product of $\sim$ 4.3 kb and combination of primers R3 and R1 (*Δasd*) yielded a PCR product of $\sim$ 5.2 kb. The PCR fragments were treated (20 min, 37 $^{\circ}$ C) with T4 DNA polymerase in the presence of 100 μ M dNTPs, subjected to agarose gel electrophoresis and the fragments eluted utilizing the commercial GeneClean kit (Bio101). The fragments were then ligated either to the 1526 bp blunt-ended Gm-resistance-conferring *Eco*RI-*Hind*III fragment from pFGS1 (Table 1) or the 1714 bp blunt-ended Gm-resistance-conferring *Bam*HI fragment from pPS716 (Table 1) to yield plasmids pPS730 (Δ *asd*: Ω Gm^rFRT), pPS729 (*Δasd*: Ω Gm^rFRT) and pPS727 [Δ (*asd*-*orfA*): Ω Gm^rFRT]. The mutated regions were then subcloned as *Bam*HI-*Nde*I fragments into pPS731 which contains the *asd* region cloned as a 6.5 kb blunt-ended *Eco*RI fragment into the *Sma*I site of the gene replacement vector pEX100T (Schweizer & Hoang, 1995). This procedure yielded plasmids pPS734 (*Δasd*: Ω Gm^rFRT), pPS738 (*Δasd*: Ω Gm^rFRT) and pPS735 [Δ (*asd*-*orfA*): Ω Gm^rFRT]. For gene replacement, the previously described strategy was employed (Schweizer & Hoang, 1995), except that Gm^r colonies were always selected, screened and maintained on VBMM-Gm media containing high concentrations (1 mg ml⁻¹) DAP. Sucrose-resistant colonies were obtained and tested for DAP requirement and auxotrophies to methionine, threonine and lysine. The mutations in the strains PAO183 [Δ (*asd*-*orfA*): Ω Gm^rFRT], PAO184 (*Δasd*: Ω Gm^rFRT) and PAO185 (*Δasd*: Ω Gm^rFRT) obtained by this procedure were verified by genomic Southern analyses (data not shown).

SmaI
 CCCGGGAAATGGCGGATGCCGTAGTCGCGGCACCTCAAGAATCTGTAACTCTGACGGGCTTGGCCGG 65
 T R E M G D A V V A A L K N L * -----
 leuB-> <-R3
 CGCCATGATGGCTGCCGGCAGCTCATTCTCTCATATAGGTAGTTCGGATGAAGCGTGTAGG 130
 ---> <----- RBS M K R V G 5
 TCTGATCGGTGGCGCGGGATGCTGGTTCCTACTCATCGACGAATGCTGGAAGAGCGGGACT 195
 L I G W R G M V G S V L M Q R M L E E R D 26
 TCGACCTGATCGAGCGGTGTCTTCCACACGCTCGAAGCTCGGTGGCCAGGCGCCGGAAGTGGCG 260
 F D L I E P V F F T T S N V G G Q G P E V G 48
 AAGGACATCGCTCCGCTGAAGACGCTACAGCATCGACGAGCTGAAGACCTCGACGTGATCCT 325
 K D I A P L K D A Y S I D E L K T L D V I L 70
 P1->
 GACCTGGCAGGCGCGGACTACACGAGCAAGTATTCCTCAAGCTGGCGGAGCGCGGTGGCAGG 390
 T C Q G G D Y T S E V F P K L R E A G W Q 91
 ClaI
 GCTACTGATGATCGATCGCCCTCCAGTCTCGCATCGAAGACGACGCGGTGATCGTCTCGACCGG 455
 G Y W I D A A S S L R M E D D A V I V L D P 113
 HindII
 GTCAACCGCAAGGTCTACGACGAGCTCTGGACGCGCGGACCCGCAACTACATCGGCGCAACTG 520
 V N R K V I D Q A L D A G A F T R N Y I G L N C 135
 CACCGTCAGCTGATGCTGATGGCTGGCGGGCTGTTCGACGCGGTCTGGTCTGATGATGA 585
 T V S L M L M A L G G L F D A G L V E W M 156
 <-P2
 GTGCCATGACCTACGAGCGGCTCCGCGCGCGCGCAGAACATGCGTGATCTGCTCAAGCAG 650
 S A M T Y Q A A S G A G A F N M R D L K L K Q 178
 NsiI
 ATGGCGCGCGCCATGCGATCGGTGGCGGATGACCTGGCAACCGCGGAGCGGATCTCGACAT 715
 M G A A H A S V A D D L A N P A S A I L D I 200
 CGACCGCAAGTGGCGGAGACCTCGCAGCGAAGCCTTCCCAACGCAATTCGGCGCGCGCG 780
 D R K V A E T L A S G A F P T E R H F G A P 221
 TGGCGCGGAGCTGATCCCTGGATCGCAAGAACTGTCCCAACGAGAGCAGAGCGCGAAGAG 845
 L G G S L I P W I D K E L S Q R R Q S R E E 243
 TGAAGCGCGGAGGAGCAACAAAGATCTTCCCGCTCAAGAACCGGATCCCGCTCGAGCGG 910
 W K A Q A E T N L A G A F K N P I P V D G 265
 TATCTGCGTGGCGTGGCGCGCATGCGTGGCCAGCGCGCTGACCATCAAGTTGAACAAG 975
 I C V R V G A M R C H S Q A L T I K L N K 286
 ATGTCGCGCTGACCGACATCGAGGCGCTGATCAGACGACAACTCCCTGGTCAAGCTGGTGGC 1040
 D V P L T D I E L G A G A F N P W V K L V G 308
 AACCCCGCGAGGTAAAGCTGCGCGAGCTGACCCCGCGCGCTCACCGGACCTGAGCGTTC 1105
 N H R E V S V R E L T P A A V T G T L S V P 330
 GGTGGCGCGCTGCGCAAGTTGAACATGGTCTCCAGTATCTCGCGCGCTTCCCGTGGCGGACC 1170
 V G R L R K L N M V S Q Y L G A F T V G D 351
 ClaI
 AGTTGCTGTGGCGCGTCCGAGCGCTGCGCGCATGTTGCGCATCTGCTGGAGCGCTGATCG 1235
 Q L L W G A A E P L R R M L R I L L E R * 371
 R1->
 ATCCGCGCGCGTCCGCAATGGCGCGCGCTCGATTGCTGGCGAAGTGGCGAAGTAAAGTGCC 1300
 <----->
 ACGCCTATTTCGACAGCGCGCGGAGCTTTTCCCATCTCCAGCGCCCTCAATGTCGAGTCTGAGG 1365
 RBS M S Q P L N V A V V G 11
 CGCCACCGGATCGGTGGCGAGGCTCTGGTCTCTCGACGAAACGCGACTTCCCGCTTCATC 1430
 A T G S V G E A L P L A G A F D R D F P L H 32
 GTTTCGACCTGCTGGCAGCGCGAGTGGCGCGGCGAGCGATGGGTTTCGCCGAAGCAGCGCTG 1495
 R L H L L A S A E S A G Q R M G F A E S S L 54
 PU->
 CGCTGCGCGGATGTCGATAGCTTCGATTTTCCAGTGTGCGGCTGGCCTTCTCGCGCGCGAGC 1560
 R V G D V D S F D T S S V G L A F F A A A A 76
 CGAGGTGTGCGGTGCCATGCGGAGCGCGCGCGCGCGGGTGTTCGCTGATCGACCTGAGCG 1625
 E V S R A H A E R A R A A G C S V I D L S 97
 GCGCCCTGGAACCGAGCGTCGACCGCGCGGTCTGATGTTTCGGTGAATCCGGAACGCTTGGCCTG 1690
 G A L E P S V A P P V M V S V N A E R L A S 119
 CAAGCGCGCGCTTCTTATTGTCCAGTCTTTCGCGCGTAGCGCGCAACTGTGCGAAGTGTGG 1755
 Q A A P F L I V Q S L R R S R R T V R S A G 141
 CGCCACTCTGCGCACGCTGGATTGCGCGCAACTGAACCTGACAGCGCTGCTCTCGCTCTCCAGC 1820
 A T P G H A G A L P D S L P L R L Q 162
 CTGGCGCGGAGGGGTCAAGAACTCGCCGCGGAGACCGCGAAGTGTCAATGCGCGCCCCCT 1885
 P G A R R G Q G T R P A D R R T A Q C A P P 184
 GGAGCGCGCTCTCTCGACCGCGAGATCGCTTCAACCTGCTCGCGAGGTGCGAGCGCGGGATG 1950
 G A S L R P P P P A R A G R S G G C 206
 CCGAGGACACAGCGCATCGACGCGCATCTTCGCGGAGGTGAGGCTTCTTGGCGAGCGT 2015
 R G T Q R D R A A H L R G G A G L A W R A 227
 PstI
 ATCGGACCGTTGAACGTATCGATCCAGGCGCGGCTATTCTTCGCGGATAGCTCAGCGTGAC 2080
 Y R T V E R Y L P G G A G I L R R * 244
 CCTGCACTGCGGAAACCGGTGGAGCTGGCGCGGTGACGCGGGTCTCGATGCGACGAAAGGTA 2145
 TCGAATGGTTCGGCGAGGCGGATTCGACGCGTGGTGGCGATGCCCTCGGCGAGGACGAGACC 2210
 TATGTGCGCGGGTGAGGCGGGGAGGCTGATCCCTGTCAAGTCAATTTGTGATTTGTCTGA 2275
 TAATGTGCGAAAGGTGCCGCGTGAATCCGCTGCTCTGGTGAGTTGTGATAAACACTATC 2340
 R2->
 TGTAAAGAGACTTACCCACAGATTGTAGAAATTATTCGAGCTTGTTAATCTGGTTTCGATCCGC 2405
 * Stop NsiI
 TGTGCGCGGGAGCGTCCGTCATG 2433

RESULTS AND DISCUSSION

Cloning of the *asd* gene of *P. aeruginosa*

The partial *asd* coding sequence was successfully amplified from *P. aeruginosa* chromosomal DNA by employing two oligonucleotide primers (P1 and P2; marked in Fig. 3) with minor degeneracy modelled after conserved amino acid sequence regions found in all Gram-negative Asd proteins cloned and sequenced to date. Under the experimental conditions employed in this study, amplification was very specific and the 281 bp amplified DNA fragment comprised > 99% of the observed PCR products (data not shown). With the help of the PCR-generated, cloned DNA fragment, the entire *asd* gene was cloned utilizing the strategy described in Methods. The physical maps of some representative *asd* clones are shown in Fig. 2. Plasmid pPS412 DNA when used to transform Δ *asd* strain SH309 was able to complement the *asd* mutation of this strain. Deletion of a 1.4 kb *SmaI* fragment (pPS455) or 1.2 kb *Bam*HI-*Pst*I fragment (achieved by subcloning of the *SmaI*-*Pst*I fragment from pPS455 to form pPS456) had no effect on *asd* complementing activity, localizing the *asd* gene between the *Pst*I and *Sma*I sites. Finally, partial digestion with *Cla*I and subcloning of a 1.2 kb *Cla*I-*Sma*I fragment into pBluescript SK localized *asd* between these sites on pPS457. This location was verified by deletion of the 0.8 kb *Cla*I fragment and replacement with a *Gm*^r marker (pPS452). The resulting pPS452 could no longer complement the *asd* deletion of SH309.

Sequence analysis of the *asd* gene region

To verify that the cloned gene fragment contained *asd*, the nucleotide sequence of a 2433 bp *Sma*I-*Nsi*I fragment was determined (Fig. 3). The sequence defines three ORFs extending from nt 1 to 44, nt 117 to 1229 and nt 1334 to 2065, which are separated by short intergenic regions. Although both of these contain palindromic sequences that could possibly serve as Rho-independent transcription termination signals (Rosenberg & Court, 1979), no promoter sequences are readily apparent.

Fig. 3. Nucleotide sequence of the cloned *P. aeruginosa asd* region. The nucleotide sequence of a *Nsi*I-*Sma*I fragment (Fig. 2) is shown. PCR-amplified chromosomal sequences (nt 330-608) are marked by the inverted arrows above the sequence. The predicted amino acid sequences of the *asd*, *leuB* and *orfA* reading frames are indicated below the appropriate codons. The putative ribosome binding sites (RBS) are marked. The amino acid residues constituting part of the Asd active site are double-underlined. Sequences located in the *leuB*-*asd* and *asd*-*orfA* intergenic regions that may serve as Rho-independent transcriptional terminators for *leuB* [$\Delta G = -31.7$ kcal mol⁻¹ (-132.6 kJ mol⁻¹)] or *asd* [$\Delta G = -27.5$ kcal mol⁻¹ (-115.06 kJ mol⁻¹)], respectively, are underlined with the inverted arrows. The location of priming sites for PCR primers mentioned in the text are underlined and the 5' to 3' direction of synthesis from these primers is indicated by the arrow next to the primer label. A vertical slash labelled 1673 marks the location of nucleotide G1673 that was arbitrarily deleted to extend OrfA to the indicated stop codon (nt 2343-2345) and to achieve the alignments to *Vibrio* spp. Asd proteins are discussed in the text.

P.a. 1	MKRVGLTGWRGMVGSVLMQRMLEERDFDLIPVFFFT	36
H.i. 1	MKNVGFIGWRGMVGSVLMQRMSEENDFENLNPVFFFT	36
E.c. 1	MKNVGFIGWRGMVGSVLMQRMVEERDFDAIPVFFFS	36
P.a. 37	TSNVGAQGFPEVDKDIPFLKDAYSIDELKTLDMVLLT	71
H.i. 37	TSQAGGKAPVFGGKDAAGDLKSAFDILELKKLDIIVT	72
E.c. 37	TSQLGGAAPSFVGGTGTGTLQDAFDLEALLKALDIIVT	71
P.a. 72	CQGGDYTSSEVFPKLRAGWQGYWIDAASSLRMEDDA	107
H.i. 73	CQGGDYTNVYPKIKATIGWDGYWIDAASSLRMKDDA	108
E.c. 72	CQGGDYTNETIYPKLRISGWQGYWIDAASSLRMKDDA	107
P.a. 108	VIVLDPVNRKVIDQALDAGTRNYIIGGNCVTSLMLMA	143
H.i. 109	TIIVLDPVNQHVISEGLKKGLKTFVGGNCVTSLMLMA	144
E.c. 108	LIITLDPVNDIVITDGLNNGIRTFVGGNCVTSLMLMS	143
P.a. 144	LGGLFDAGLVEWMSAMTYQAASGAGAQNMRLDLKQM	179
H.i. 145	IJGLFEKDLVEWISVATYQAASGAGAKNMRELLSQM	180
E.c. 144	LGGLFANDLVDWVSVATYQAASGAGARHMRRELLTQM	179
P.a. 180	GAAHASVADDLANPASAILDIDRKVAETLRSEAFPT	215
H.i. 181	GLLEQAVSSELKOPASSILDIERKVTAKMRADNFT	216
E.c. 180	GHLVYGHVADELAITSSAILDIERKVTTLTRSGELP	215
P.a. 216	EHFGAPLGGSLIPWIDKELISQRHQSREEWKAGQETN	251
H.i. 217	DNFGAALGGSLIPWIDKLLPETGQTKKEWKGYAETN	252
E.c. 216	DNFGVPLAGSLIPWIDKQLDNIGQSREEWKGAETN	250
P.a. 252	KILAREKNPIPVGDLCVRVGAMRCHSQAFTIKLKKD	287
H.i. 253	KILGLSDNPIPVGDLCVRIGALRCHSQAFTIKLKKD	288
E.c. 251	KILNTSSV-LIPVDGLCVRVGALRCHSQAFTIKLKKD	285
P.a. 288	VPLTDITGLIRQHNPVWKLLVPNHRELSVRELTPAAV	323
H.i. 289	LPLEEELQILASHNWEWVKVLPNDKEITLRELTPAKV	324
E.c. 286	VSIPTVIEELLIAHNPNWAKVVPNDREITMRELTAAV	321
P.a. 324	TGTLSPVPVGRRLKLNMSQYLGAFVGDQQLWGAAE	359
H.i. 325	TGTLSPVPVGRRLKLMGPEYLAFAFTVGDQQLWGAAE	360
E.c. 322	TGTLTTPVGRRLKLNMGPEFLSFAFTVGDQQLWGAAE	357
P.a. 360	PLRRMLRIJLLER	371
H.i. 361	PVRRLLKQLVA	371
E.c. 358	PLRRMLRQLA	367

Fig. 4. Amino acid sequence alignment generated utilizing the SeqVu program (Garvan Medical Institute, Sidney, Australia). Alignments of Asd of *P. aeruginosa* (P.a.) with Asd from *H. influenzae* (H.i.) (GenBank accession number L42023) and *E. coli* (E.c.) (GenBank accession number V00262) are shown. The similarities of the Asd proteins are 82% (P.a. vs H.i.) and 77% (P.a. vs E.c.).

However, in the absence of transcriptional analyses no definitive conclusions about the transcriptional arrangements of the genes encoding these ORFs can be drawn.

The shortest ORF extends from nt 1 to 44 and was previously shown to constitute the C-terminal part of the *P. aeruginosa* LeuB homologue (Hoang & Schweizer, 1997).

The longest ORF is encoded by nt 117–1229 and encodes 371 residues with a combined molecular mass of 40917. BLAST and PROSITE searches revealed that this protein shares significant identity to other Asd proteins (Fig. 4). Alignments of the predicted amino acid sequence of Asd from *P. aeruginosa* with the corresponding sequences from *E. coli* and *Haemophilus influenzae* revealed greater than 80% similarity and for long stretches the sequences align perfectly. In contrast, when compared to the Asd proteins from *Bacillus subtilis* (GenBank accession number Z22554), *Streptococcus mutans* (Cardineau & Curtiss, 1987) and *Vibrio cholerae* (GenBank accession number X55363), much less similarity exists between the deduced amino acid sequences of either Asd proteins (data not shown). While it is not surprising that the Asd sequences from Gram-negative bacteria are more conserved amongst each other as compared to Gram-positive bacteria, it is surprising that

Asd from *V. cholerae* shows little homology to Gram-negative sequences but higher homology to Gram-positive sequences.

A second ORF extends from nt 1334 to 2065 and encodes 244 amino acid residues specifying a polypeptide with a calculated molecular mass of 25543. BLAST analyses indicated that the first 114 residues of this protein had significant similarity to the Gram-positive-like Asd proteins, especially the Asd proteins from several *Vibrio* species. Since this homology did not extend beyond residue 114 and since a closer inspection of the residual OrfA coding sequence revealed a codon usage somewhat atypical of *P. aeruginosa* (West & Iglewski, 1988), we considered the possibility of a frameshift mutation contained on the cloned and sequenced DNA fragments. This notion was further supported by the fact that arbitrary deletion of a single G residue at position 1673 (Fig. 3) allowed translation of the same ORF to a TAA stop codon at nt 2343–2345. This ORF would contain at least 336 amino acid residues with a calculated molecular mass of 35611. Thus, it would be very similar in size and amino acid composition to the Asd proteins from selected *Vibrio* species.

To rule out the possibility of a frameshift mutation contained on the cloned and sequenced DNA fragments, two primers (PU and PD; Fig. 3) were designed, and the intervening region was PCR-amplified from genomic PAO1 DNA and sequenced. The sequence thus obtained was identical to the one determined from the cloned DNA segments. Furthermore, PCR amplification and nucleotide sequence analysis of the same region from genomic DNA of another unrelated *P. aeruginosa* strain, UO606, which is a *glpK*::Tn5Gm derivative of the prototrophic strain PG201 (Ochsner *et al.*, 1994), yielded an identical sequence. Therefore, it is most likely that the *orfA* region from the analysed *P. aeruginosa* strains is naturally frame-shifted and therefore does not express a protein with Asd function. This notion is further supported by several other observations: (i) non-polar and polar Gm^r insertions in chromosomal *asd* confer the same Asd⁻ phenotype; (ii) pPS457 and a similar broad-host-range derivative containing only the *asd* coding region complements the Asd phenotype of *P. aeruginosa* $\Delta(asd-orfA)$ and Δasd mutants, as well as *E. coli* Δasd (Fig. 2); and (iii) plasmids containing the cloned *asd* region in *E. coli* highly express LeuB and Asd from the *lac* promoter but neither a 26 kDa nor a 36 kDa protein (Hoang & Schweizer, 1997). Thus, the biological significance of *orfA* and its product, if any, remain unknown.

Conserved sequences of Gram-negative Asd proteins containing the active site residues

In conjunction with earlier ligand experiments, Biellmann *et al.* (1980) and Haziza *et al.* (1982) identified the sequence FVGGNCTVS as being part of the active site residues of Asd from *E. coli*. When an extended version of this sequence was used in a BLASTP search of

GenBank, similar sequences with the consensus GGNCTVXMLMXXXLGLF (double-underlined in Fig. 3) differing by only a few conserved substitutions were found in almost identical positions in the Asd proteins from all of the Gram-negative bacteria from which the sequences are available to date. Although these amino acid sequences are thought to comprise part of the active site residues, they were not readily apparent in Asd from *Vibrio* species and the Gram-positive bacteria. Thus, the above consensus sequence may be used as a signature motif for Gram-negative Asd proteins.

Identification of the *asd* gene product

To identify the Asd polypeptide, the *P. aeruginosa* wild-type *asd* gene was expressed in the T7 RNA polymerase/T7 promoter expression system (Studier *et al.*, 1990). Only pT7-*5asd* containing *asd* in the same orientation as the T7 promoter highly expressed an IPTG-inducible single protein with an estimated molecular mass of 40 kDa (data not shown). This polypeptide was not expressed from pT7-6*asd* carrying *asd* in the opposite orientation and was also absent from vector-containing cells. The observed size (40 kDa) of the *P. aeruginosa* Asd polypeptide compares very favourably with the size deduced from the nucleotide sequence (40 917), as well as the predicted sizes of 40 015 for the *E. coli* (GenBank accession number V00262) and 40 537 for the *H. influenzae* (GenBank accession number L42023) Asd proteins.

Isolation of a chromosomal *asd* mutation

As detailed in Methods, several chromosomal Δ *asd* mutations were isolated either by replacing the *asd* gene alone or by replacing the *asd-orfA* encoding sequences with different Gm-resistance-conferring cassettes. Selection of these cassettes allowed isolation of the following mutants: PAO183 [Δ (*asd-orfA*): Ω Gm^rFRT], PAO184 (Δ *asd*: Ω Gm^rFRT) and PAO185 (Δ *asd*: Ω Gm^rFRT). On VBMM minimal medium, all of these mutants were auxotrophic for DAP, lysine, methionine and threonine but grew on this medium in the presence of DAP. This indicated that the biosynthetic pathways for these amino acids (Fig. 1) are probably similar to those proposed for *E. coli* and *S. typhimurium* (Patte, 1996; Greene, 1996), with the exception of the pathway from homoserine to methionine which was shown to be different in *P. aeruginosa* (Fogliano *et al.*, 1995). The mutant required high (1 mg ml⁻¹) concentrations of DAP, and even at these levels its growth rate was slower than the one observed with its parent, PAO1. This is probably due to the fact that *P. aeruginosa* does not effectively transport DAP (Martin *et al.*, 1986), a notion which is further corroborated by the finding that even in the presence of DAP, the mutant did not grow as well on minimal medium lacking lysine as did wild-type PAO1. In the absence of DAP, the mutants lysed rapidly. All mutants were complemented by pPS600 containing the *asd* gene

cloned in the broad-host-range vector pUCP20T (Schweizer *et al.*, 1996).

Chromosomal mapping of the cloned *asd* gene

The *asd* gene was located on the PAO1 chromosome by hybridization of labelled pPS410 to Southern blots of *DpnI*- and *SpeI*-digested genomic DNA, which previously had been separated by PFGE (Lightfoot & Lam, 1993) (data not shown). In blots of *SpeI*-digested chromosomal DNA, the *asd* sequences hybridized to a restriction fragment of approximately 260 kb, corresponding to restriction fragment *SpeI*-I (Holloway *et al.*, 1994). Similarly, the same probe hybridized to a *DpnI* fragment of approximately 580 kb, which corresponds to fragment *DpnI*-D. Thus, the *asd* gene was mapped to between 1.85 and 2.15 Mbp on the 5.9 Mbp chromosome, which corresponds to the 37–40 min region of the genetic map (Holloway *et al.*, 1994). The results demonstrate that *P. aeruginosa* contains a single copy of the *asd* gene. Interestingly, the *asd* gene, whose function is required for lysine, methionine and threonine biosynthesis, maps to the same macrorestriction fragments as some other genes involved in amino acid biosynthesis and/or metabolism, for example *leu-10*, *trpF*, *met-9011* and *oru-314* (Holloway *et al.*, 1994), and we have recently shown that *leu-10* is allelic with *leuB*, encoding 3-isopropylmalate dehydrogenase (Hoang & Schweizer, 1997).

Conclusions

The results described in this paper demonstrate that the *asd* gene encoding aspartate- β -semialdehyde dehydrogenase from *P. aeruginosa* was successfully cloned and expressed. Although the *asd* downstream region contains a putative gene, *orfA*, whose product showed significant homology to the Gram-positive-like Asd proteins from *Vibrio* spp., molecular and genetic analyses indicated that *orfA* is naturally frame-shifted in all *P. aeruginosa* strains tested so far. Thus, its physiological role, if any, in DAP biosynthesis remains to be elucidated. Chromosomal mapping studies and mutational analyses indicated that *P. aeruginosa* contains a single copy of *asd*. However, this does not rule out the existence of a second (silent) gene encoding an Asd-like activity elsewhere in the chromosome, which may be revealed by reversion analysis of the *asd* insertion mutants described in this study. The anticipated role of *asd* in the biosynthetic pathways of DAP, lysine, methionine and threonine (Fig. 1) was supported by mutational analyses. The studies also indicated that *asd* is not closely linked to any of the hitherto unidentified aspartokinase (*ask*) gene(s).

It is anticipated that the availability of the cloned gene will facilitate future genetic studies of this organism based on exploitation of the essential nature of *asd*. Since *asd* mutants have an obligate requirement for DAP, in the laboratory they will only grow on media supplemented with DAP, or after acquiring the *asd* gene

on a complementing DNA fragment. The latter can be exploited for *in vivo* maintenance of recombinant plasmids containing an 'asd cassette' as the selectable marker in a genetically engineered *asd* deletion background. In addition, it is anticipated that this will provide a powerful means for isolation and selection of defined chromosomal insertion and deletion mutants. Given the problems with using antibiotic resistance markers in this bacterium, such a 'metabolic' genetic element based on an essential bacterial gene presents an attractive alternative method for gene manipulation and analysis. Finally, one could imagine *asd* as an alternative selectable marker for the emerging *in vivo* expression technology (IVET) strategies (Slauch *et al.*, 1994; Wang *et al.*, 1996). Given that, in contrast to some of the other selectable markers present on currently available constructs, it is 'bacteria-specific', *asd* may offer a better alternative. To facilitate such studies our laboratory has already (i) isolated a defined $\Delta(\text{asd-orfA})$ *P. aeruginosa* host strain devoid of any antibiotic resistance marker by employing FLP-recombinase-mediated excision (Cherpanov & Wackernagel, 1995) of the $\Omega\text{Gm}^r\text{FRT}$ cassette contained in strain PAO183 $\Delta(\text{asd-orfA})::\Omega\text{Gm}^r\text{FRT}$ and (ii) genetically engineered a defined *asd* cassette for use in vector construction.

ACKNOWLEDGEMENTS

S.W. was supported by a Summer Studentship from the Alberta Heritage Foundation for Medical Research. Financial support to H.P.S. was provided by a Medical Scholarship of the Medical Research Council (MRC) of Canada, MRC operating grant MT-11245, by funds from the Canadian Bacterial Diseases Network, and by start-up funds from Colorado State University.

REFERENCES

- Biellmann, J. P., Eid, P., Hirth, C. & Joernvall, H. (1980). Aspartate- β -semialdehyde dehydrogenase from *Escherichia coli*. Affinity labeling with the substrate analogue L-2-amino-4-oxo-5-chloropentanoic acid: an example of half-site reactivity. *Eur J Biochem* **104**, 59–64.
- Bodey, G. P., Bolivar, R., Fainstein, V. & Jadeja, L. (1983). Infections caused by *Pseudomonas aeruginosa*. *Rev Infect Dis* **5**, 279–313.
- Cardineau, G. A. & Curtiss, R. (1987). Nucleotide sequence of the *asd* gene of *Streptococcus mutans*. *J Biol Chem* **262**, 3344–3353.
- Chamberlain, J. P. (1979). Fluorographic detection of radioactivity in polyacrylamide gels with the water-soluble fluor, sodium salicylate. *Anal Biochem* **98**, 132–135.
- Cherpanov, P. P. & Wackernagel, W. (1995). Gene disruption in *Escherichia coli*: Tc^R and Km^R cassettes with the option of FLP-catalyzed excision of the antibiotic-resistance determinant. *Gene* **158**, 9–14.
- Clepet, C., Borne, F., Krishnapillai, V., Baird, C., Patte, J. C. & Cami, B. (1992). Isolation, organization and expression of the *Pseudomonas aeruginosa* threonine genes. *Mol Microbiol* **6**, 3109–3119.
- Cryz, S. J., Jr, Pitt, T. L., Furer, E. & Germaier, R. (1984). Role of lipopolysaccharide in virulence of *Pseudomonas aeruginosa*. *Infect Immun* **44**, 508–513.
- Fogliano, M., Borne, F., Bally, M., Ball, G. & Patte, J. C. (1995). A direct sulfhydrylation pathway is used for methionine biosynthesis in *Pseudomonas aeruginosa*. *Microbiology* **141**, 431–439.
- Govan, J. R. W. (1988). Alginate biosynthesis and other unusual characteristics associated with the pathogenesis of *Pseudomonas aeruginosa* in cystic fibrosis. In *Bacterial Infections of Respiratory and Gastrointestinal Mucosa*, pp. 67–96. Edited by W. Donachie, E. Griffiths & J. Stephen. Oxford: IRL Press.
- Greene, R. C. (1996). Biosynthesis of methionine. In *Escherichia coli and Salmonella*, pp. 542–560. Edited by F. C. Neidhardt, R. Curtiss, III, J. L. Ingraham and others. Washington, DC: American Society for Microbiology.
- Haziza, C., Stragier, P. & Patte, J.-C. (1982). Nucleotide sequence of the *asd* gene of *Escherichia coli*: absence of a typical attenuation signal. *EMBO J* **1**, 379–384.
- Hoang, T. T. & Schweizer, H. P. (1997). Leucine biosynthesis in *Pseudomonas aeruginosa*: identification and characterization of *leuB*, encoding 3-isopropylmalate dehydrogenase. *Mol Gen Genet* (in press).
- Hoiby, N., Pedersen, S. S., Shand, G. H., Doering, G. & Holder, I. A. (1989). *Pseudomonas aeruginosa* infection. *Antibiot Chemother* **42**, 1–300.
- Holder, I. A. & Neely, A. N. (1991). The role of proteases in *Pseudomonas* infections in burns: a current hypothesis. *Antibiot Chemother* **44**, 99–105.
- Holloway, B. W. & Zhang, C. (1990). Genetic maps. In *Locus Maps of Complex Genomes*, pp. 2.71–2.78. Edited by S. J. O'Brien. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Holloway, B. W., Römling, U. & Tümmler, B. (1994). Genomic mapping of *Pseudomonas aeruginosa* PAO. *Microbiology* **140**, 2907–2929.
- Kureishi, A. & Bryan, L. E. (1992). Pre-boiling high GC content, mixed primers with 3' complementation allows the successful PCR amplification of *Pseudomonas aeruginosa* DNA. *Nucleic Acids Res* **20**, 1155.
- Lightfoot, J. & Lam, J. S. (1993). Chromosomal mapping, expression and synthesis of lipopolysaccharide in *Pseudomonas aeruginosa*: a role for guanosine diphospho (GDP)-D-mannose. *Mol Microbiol* **8**, 771–782.
- Liss, L. (1987). New M13 host: DH5 α F' competent cells. *Focus* **9**, 13.
- Makowski, G. S. & Ramsby, M. L. (1993). pH modification to enhance the molecular sieving properties of sodium dodecyl sulfate-10% polyacrylamide gel. *Anal Biochem* **212**, 283–285.
- Martin, C., Cami, B., Jeenes, D. D., Haas, D. & Patte, J.-C. (1986). Heterologous expression and regulation of the *lysA* gene of *Pseudomonas aeruginosa* and *Escherichia coli*. *Mol Gen Genet* **203**, 430–434.
- Miller, J. H. (1992). *A Short Course in Bacterial Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Nakayama, K., Kelly, S. M. & Curtiss, R. (1988). Construction of an Asd⁺ expression-cloning vector: stable maintenance and high level expression of cloned genes in a *Salmonella* vaccine strain. *BioTechnology* **6**, 693–697.
- Ochsner, U. A., Koch, A. K., Fiechter, A. & Reiser, J. (1994). Isolation and characterization of a regulatory gene affecting rhamnolipid synthesis in *Pseudomonas aeruginosa*. *J Bacteriol* **176**, 2044–2054.
- Patte, J.-C. (1996). Biosynthesis of threonine and lysine. In *Escherichia coli and Salmonella*, pp. 528–541. Edited by F. C.

- Neidhardt, R. Curtiss, III, J. L. Ingraham and others. Washington, DC: American Society for Microbiology.
- Rosenberg, M. C. & Court, D. (1979). Regulatory sequences involved in the promotion and termination of RNA transcription. *Annu Rev Genet* 13, 319–353.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Schleifer, K. H. & Kandler, O. (1972). Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriol Rev* 36, 407–477.
- Schweizer, H. P. (1991). The *agmR* gene, an environmentally responsive gene, complements defective *glpR*, which encodes the putative activator for glycerol metabolism in *Pseudomonas aeruginosa*. *J Bacteriol* 173, 6798–6806.
- Schweizer, H. P. & Hoang, T. (1995). An improved system for gene replacement and *xylE* fusion analysis in *Pseudomonas aeruginosa*. *Gene* 158, 15–22.
- Schweizer, H. P. & Po, C. (1994). Cloning and nucleotide sequence of the *glpD* gene encoding *sn*-glycerol-3-phosphate dehydrogenase from *Pseudomonas aeruginosa*. *J Bacteriol* 176, 2184–2193.
- Schweizer, H., Sweet, G. & Larson, T. J. (1986). Physical and genetic structure of the *glpD*–*malT* interval of the *Escherichia coli* K-12 chromosome. *Mol Gen Genet* 202, 488–492.
- Schweizer, H. P., Klassen, T. R. & Hoang, T. (1996). Improved methods for gene analysis and expression in *Pseudomonas*. In *Molecular Biology of Pseudomonads*, pp. 229–237. Edited by T. Nakazawa, K. Furukawa, D. Haas & S. Silver. Washington, DC: American Society for Microbiology.
- Slauch, J. M., Mahan, M. J. & Mekalanos, J. J. (1994). *In vivo* expression technology for selection of genes specifically induced in host tissues. *Methods Enzymol* 235, 481–492.
- Studier, F. W., Rosenberg, A. H., Dunn, J. J. & Dubendorff, J. W. (1990). Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol* 185, 60–89.
- Tabor, S. (1994). Expression using the T7 RNA polymerase/promoter system. In *Short Protocols in Molecular Biology*, pp. 16.1–16.10. Edited by F. M. Ausubel, R. Brent, R. E. Kingston and others. New York: John Wiley.
- Vasil, M. L. (1986). *Pseudomonas aeruginosa*: biology, mechanisms of virulence, epidemiology. *J Pediatr* 108, 800–805.
- Vasil, M. L., Graham, M. L., Ostroff, R. M., Shortridge, V. D. & Vasil, A. I. (1991). Phospholipase C: molecular biology and contribution to the pathogenesis of *Pseudomonas aeruginosa*. *Antibiot Chemother* 44, 34–47.
- Wang, J., Mushegian, A., Lory, S. & Jin, S. (1996). Large-scale isolation of candidate virulence genes of *Pseudomonas aeruginosa* by *in vivo* selection. *Proc Natl Acad Sci USA* 93, 10434–10439.
- West, S. E. H. & Iglewski, B. H. (1988). Codon usage in *Pseudomonas aeruginosa*. *Nucleic Acids Res* 16, 9323.
- West, S. E. H., Schweizer, H. P., Dall, C., Sample, A. K. & Runyen-Janecky, L. J. (1994). Construction of improved *Escherichia-Pseudomonas* shuttle vectors derived from pUC18/19 and the sequence of the region required for their replication in *Pseudomonas aeruginosa*. *Gene* 128, 81–86.
- Wieslander, L. (1979). A simple method to recover intact high molecular weight RNA and DNA after electrophoretic separation in low gelling temperature agarose gels. *Anal Biochem* 98, 305–309.
- Xiang, C., Wang, H., Shiel, P., Berger, P. & Guerra, D. J. (1994). A modified alkaline lysis miniprep protocol using a single microcentrifuge tube. *BioTechniques* 17, 30–31.
- Yanisch-Perron, C., Vieira, J. & Messing, J. (1985). Improved M13 cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* 33, 103–119.

Received 12 July 1996; revised 16 September 1996; accepted 4 October 1996.